Scholars Academic Journal of Pharmacy (SAJP) **ISSN 2320-4206 (Online)**
Sch. Acad. J. Pharm., 2015; 4(3): 190-194 **ISSN 2347-9531 (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublisher.com

Research Article

Inhibition of Matrix Metalloproteinase-2 Expression by Ethanolic Extracts of Zingiberaceae Rhizomes in Artery Endothelial Cells

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Abstract: Atherosclerosis belongs to inflammation-related vascular diseases due to bacterial infection and causes lipid accumulation in artery wall that lead to heart attack and stroke. Matrix metalloproteinase (MMP)-2 and -9 are grouped to zinc-dependent proteases that act in degradation of extracellular matrix in atherosclerotic plaque. Instead of traditional foods, the rhizomes of Zingiberaceae have been empirically used in folk medicines purposes, including natural vascular protection. This study was aimed to test the inhibitory effect of 10 Zingiberaceae rhizomes on the expression of MMP-2 activity in lipopolysaccharide-induced artery endothelial cells by conducting gelatin zymogram. At 1 µg/ml, selected Zingiberaceae rhizomes, i.e. *Kaempferia pandurata, Curcuma xanthorrhiza, Alpinia galanga, Zingiberaceae officinale,* and *Z. officinale* Var Rubra, were found to attenuate MMP-2 activity up to 50% compared with LPS-treated cells. In summary, these data suggest that selected Zingiberaceae rhizomes may be applied for potential therapeutics in vascular protection and therapy, in particular atherotherapy.
Keywords: Zingiberaceae rhizomes, matrix metalloproteinase-2 activity, atherosclerosis, artery endothelial cells.

INTRODUCTION

Atherosclerosis is a diffuse, systemic disease of the arterial network, the local manifestations of which are associated with clinical problems such as myocardial infarction, stroke, etc. It has been reported that atherosclerotic plaque is responsible for 75% of human death [1]. The equilibrium between matrix metalloproteinase (MMP) and its endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMP), is critical in the maintenance of the cardiovascular system. MMPs are a group of zinc-dependent proteinases capable of degrading extracellular matrix protein in many physiological and pathological processes, including atherosclerosis. Among MMP groups, it has been recognized that the expression and activity of MMP-2 and MMP-9 are linearly associated with atherosclerotic plaque [2].

Zingiberaceae belongs to the ginger family and is mainly distributed in Asian regions. The rhizomes of Zingiberaceae have been empirically used for culinary and folk medicines [3]. They exerted several bioactivities, including antioxidant, anti-inflammatory, antimicrobial, and anticaries properties [4-6]. Our previous study demonstrated that most Curcuma rhizomes significantly acted as vascular protection through attenuation of MMP-9 protein and gen in human umbilical vascular endothelial cells (HUVECs) *in vitro* [7]. This study was focused on screening the effect of medicinal Zingiberaceae rhizomes on the inhibition of MMP-2 activity in artery endothelial cell system.

MATERIALS AND METHODS

Plant materials and sample preparation

Ten Zingiberaceae rhizome plants, i.e. such as *Kaempferia pandurata*, *K. galanga*, *Alpinia galanga*, *Zingiber officinale*, *Z. Officinale* Var Rubra, *Curcuma xanthorrhiza*, *C. longa*, *C. zedoria*, *C. mangga*, and *C. aeruginosa* were collected from traditional markets in Jakarta and Bogor. Samples were dried and grinded, followed by extraction two times with 70% ethanol at room temperature for 3 days each, and the combined extracts were concentrated by freeze-drying treatment (yield: 10% w/w).

Cell culture and cell viability

Bovine pulmonary artery endothelial cells (BPAE lines; ATCC CCL-209; American Type Culture Collection) were purchased from Primate Research Center, Bogor Agricultural University, Bogor. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. Cells were incubated in the presence of 5% CO_2 at 37^{o}C. The cells (passage 7-11) were seeded at a concentration of 2 x 10^5 cells/ml per 75-cm^2 flask and cultured for 24 h. Cells were then activated with

Eschericia coli O157:H7 lipopolysaccharide (LPS; Sigma-Aldrich) to enhance the production of MMP-2.

The effects of *E. coli* LPS and Zingiberaceae extracts on cell viability were evaluated with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich) colorimetric assay [7]. Zingiberaceae rhizome extract was dissolved in 100% DMSO, and the stock solution of the extract at a concentration of 1000 µg/ml was prepared in 10% DMSO. The final concentrations of the extract were 1 µg/ml in the culture media, and all cells were treated with DMSO at a final concentration of 0.1%.

Sample treatment

Cells were seeded at a concentration of 2×10^5 cells/ml in 6-well plates and cultured for 24 h in DMEM-FBS. After washing with Dulbecco's phosphate-buffered saline (DPBS), the cells were incubated in serum free-DMEM without LPS (negative control group), with 2 µg/ml LPS (positive control group), or with 2 µg/ml LPS plus treatment for 24 h. The treatment groups included Zingiberaceae rhizome extracts (1 µg/ml) and MMP inhibitor (doxycycline at 1 µg/ml). Conditioned media were collected for further experiments.

Gelatin zymogram

Activity of MMP-2 in the conditioned media was measured by gelatin zymography [7]. Briefly, the conditioned media from the negative control, positive control and treatment group (Zingiberaceae extracts) were collected and subjected to electrophoresis with 10% SDS polyacrylamide gels containing 0.1 % gelatin. Electrophoresis was run at 90 V for 1.5 h in an electrophoretic apparatus (Bio-Rad Mini Protean 3 Cell). After electrophoresis, gels were washed twice with 25 ml of 2.5% Triton X-100 on a gyratory shaker for 1 h at room temperature to remove SDS. Gel was then incubated in 25 ml reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 0.15 M NaCl) at 37ºC for 24 h, stained with Coomassie brilliant blue R-250 and destained with methanol-acetic acid in water. Briefly, MMP-2 gelatinolytic band was detected at 67 kDa as clear zone against the dark background. Relative band densities were analyzed by Gel-Doc Quantity One software (Bio-Rad Laboratories) and calculated by Multi Gauge software (Lab Science).

Statistical analysis

Triplicate experiments were performed throughout this study. All data were presented as the mean ± standard deviation (SD). The significance of differences between control and treated groups were statistically analyzed by the paired Student's *t*-test (**P* < 0.05).

RESULTS AND DISCUSSION

The viability of BPAE cells treated with LPS and Zingiberaceae rhizome extracts was determined. MTT colorimetric assay showed that LPS (2 µg/ml), Zingiberaceae rhizome extracts (1 µg/ml), and doxycycline MMP inhibitor (1 µg/ml) were safe to the BPAE cell viability (Figure 1). At 5 µg/ml, most Zingiberaceae rhizomes caused ≥20% of cell toxicity in BPAE cells. Thus, low concentration (1 µg/ml) of each extract was used for the further study.

Next, Zingiberaceae rhizomes were investigated for their anti-atherosclerotic potential via decreasing MMP-2 activity in LPS-induced BPAE cells. Zymogram profile demonstrated that BPAE cells were found to secret MMP-2 directly, and LPS treatment at 2 µg/ml significantly enhanced the production of MMP-2 activity in the cells (Figure 2).

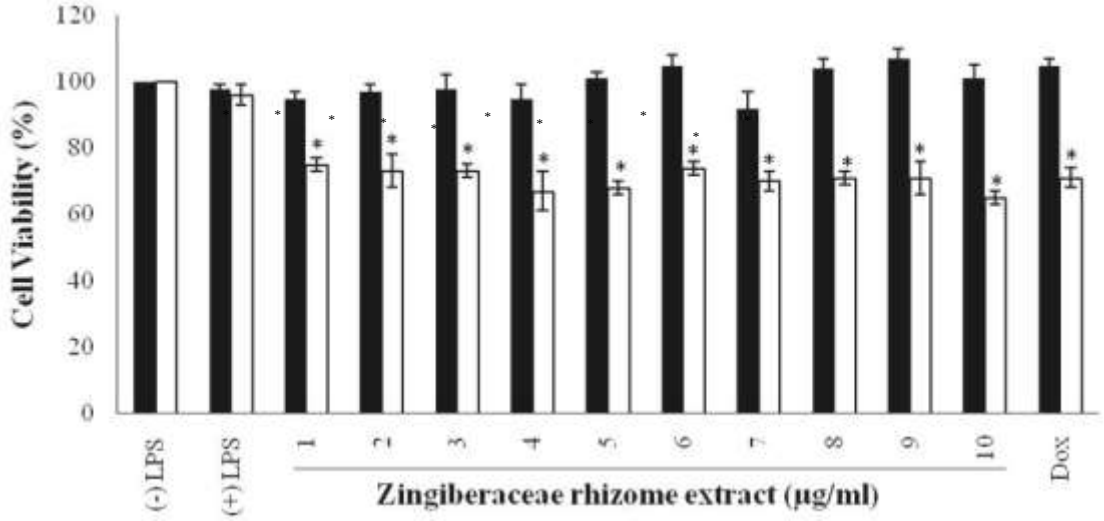

Fig-1: Effect of LPS (2 µg/ml) and Zingiberaceae rhizome extracts at 1 µg/ml (■) and 5 µg/ml (□) on the BPAE cell viability. Dox refers to doxycycline (standard MMP inhibitor) at similar concentrations. 1. *K. pandurata*; 2. *C. xanthorriza*; 3. *C. aeruginosa*; 4. *C. mangga*; 5. *C. zedoaria*; 6. *C. longa*; 7. *K. galanga*; 8. *A. galanga*; 9. *Z. officinale*; 10. *Z. officinale Roxb*. Var Rubra; Dox. Doxycycline (standard MMP inhibitor). Values represent the mean ± SD of triplicate experiments. * indicates *P* < 0.05 against LPS-treated cells.

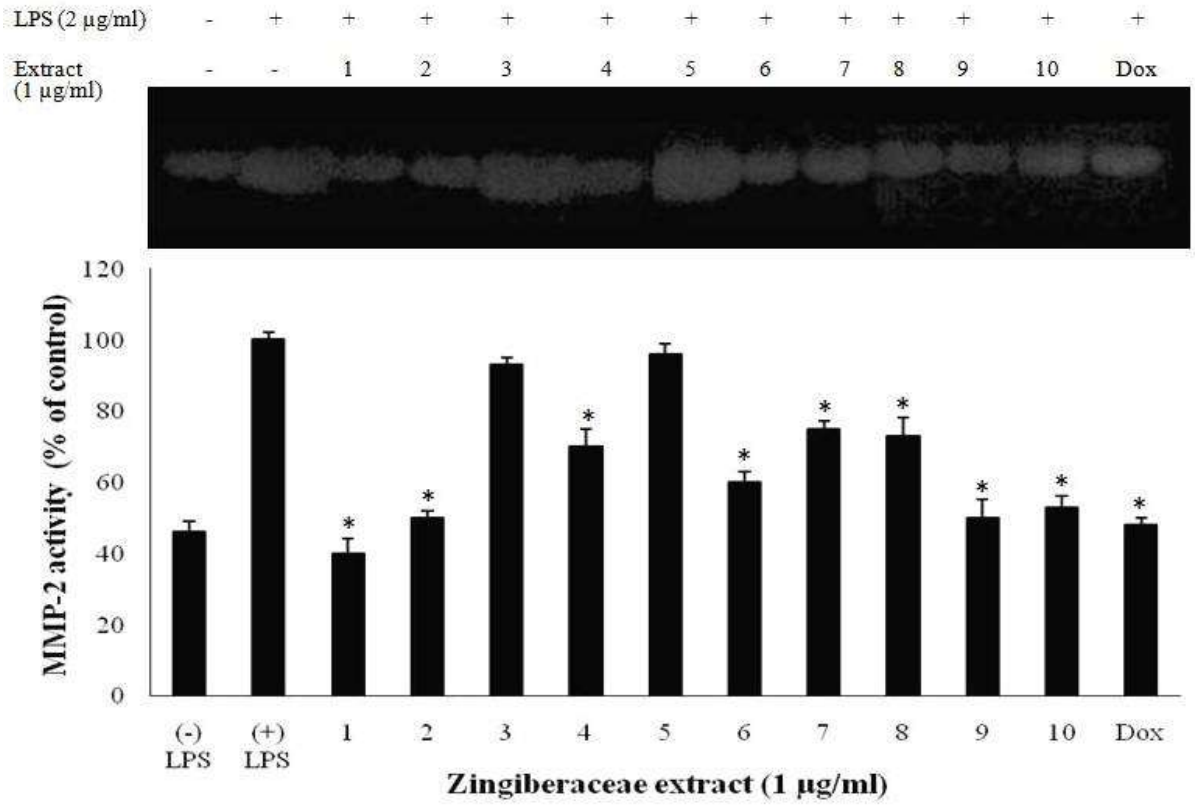

Fig-2: Effect of Zingiberaceae rhizome extracts on the expression of MMP-2 activity in LPS-induced BPAE cells assayed by gelatin zymography. 1. *Kaempferia pandurata*; 2. *Curcuma xanthorriza*; 3. *C. aeruginosa*; 4. *C. mangga*; 5. *C. zedoaria*; 6. *C. longa*; 7. *K. galanga*; 8. *Alpinia galanga*; 9. *Zingiber officinale*; 10. *Z. officinale Roxb.* Var Rubra; Dox. Doxycycline (standard MMP inhibitor). Values represent the mean ± SD of triplicate experiments. * indicates *P* < 0.05 against LPS-treated cells.

Among all, rhizome extracts of *K. pandurata*, *C. xanthorrhiza*, *A. galanga*, *Z. officinale*, and *Z. officinale* Var Rubra at 1 µg/ml were found to inhibit >50% of MMP-2 activity in LPS-induced BPAE cells. Doxycycline MMP inhibitor also showed the similar MMP-2 inhibitory activity with those selected Zingiberaceae rhizome extracts.

It has been shown that atherosclerotic plaques consisted of various vascular cells, including macrophages, endothelial, and smooth muscle cells within an accumulation of lipid and extracellular matrix proteins [8]. The use of an infectious agent of LPS is reported to be involved in the formation of atherosclerotic plaque through triggering the production of MMPs particularly MMP-2 and MMP-9, suggesting that bacterial infection may associate with the initiation of atherosclerosis [9].

In accordance with our study, LPS at 2 µg/ml was found to enhance MMP-2 secretion in vascular cell type of BPAE, whereas the cells also primarily produced MMP-2 activity (Figure 2). In addition, other vascular stimuli such as thrombin, interleukin (IL)-1α, and tumor necrosis factor (TNF)-α also up-regulated MMP-2 and MT-MMP production in human aortic vascular smooth muscle cells [10-11].

Zingiberaceae or ginger family has been known for their potentials in vascular therapy due to their antioxidative, anti-inflammatory, and lowering effects on LDL. These properties are believed in association with prevention and treatment of atherosclerosis [12-13]. The use of Zingiberaceae rhizomes for screening a natural MMP inhibitor derived from plants is assumed to be mainly correlated with their secondary metabolite contents. Most bioactive compounds derived from Zingiberaceae rhizomes are grouped in polyphenols, terpenes, and essential oils and have been reported for their multi-pharmacological effects.

Curcumin from *C. longa*, xanthorrhizol from *C. xanthorrhiza*, panduratin A from *K. pandurata*, and licarin A from *C. zedoria* possessed various MMP-1, MMP-2, and MMP-9 inhibitory properties in several *in vitro* cell culture assays [14-19]. Extracts of Taiwanese Zingiberaceae of *A. pricei*, *C. zedoria*, *C. longa*, and *K. pandurata* were also found to reduce MMP-2 and MMP-9 activities in various *in vitro* disease models of cancer, tumor, periodontitis, and atherosclerosis [20-23].

In line with this study, our previous findings indicated that most Curcuma rhizome extracts, i.e. *C.*

xanthorrhiza, C. mangga, C. longa, at 1 µg/ml significantly reduced the expression of MMP-9 activity in HUVECs exposed to LPS *in vitro* [7]. Meanwhile, in vascular artery endothelial cell system, 5 Zingiberaceae rhizomes (*K. pandurata, C. xanthorrhiza, A. galanga, Z. officinale,* and *Z. officinale* Var Rubra) at 1 µg/ml significantly reduced MMP-2 activity when compared with LPS treatment (Figure 2). However, the inhibitory molecular mechanism of specific Zingiberaceae rhizome extracts on MMP-2 expression in LPS-induced BPAE cells still remains unclear.

Previous studies summarized that plant polyphenols and wine polyphenols may have spesific short- and long-term roles in vascular protection via modulation nitric oxide-mediated vasorelaxation, the increased expression of endhothelial nitric oxide synthase, the decreased expression of adhesion molecules and growth factors, the involvement of cell migration and proliferation, and the inhibition of MMPs involved in the degradation of extracellular matrix proteins [24-26]. Hence, our data suggest that specific medicinal Zingiberaceae rhizomes with potential MMP-2 inhibitory effect may exert long-term mechanisms on lowering the risk of vascular diseases including atherosclerosis

Doxycycline, a standard MMP inhibitor, was also tested in this study for comparison. Doxycycline at 1 µg/ml demonstrated the similar efficacy with those 5 selected rhizomes for ameliorating MMP-2 activity in LPS-induced BPAE cells (Figure 2). These findings are also in linear with Mannacio *et al.* [27]. The specific role of doxycyline in inhibiting MMP-2 and MMP-9 levels is thought to be associated with the prevention of graft atherosclerosis and vascular remodeling.

CONCLUSION

Certain Zingiberaceae rhizome exctracts, i.e. *K. pandurata, C. xanthorrhiza, A. galanga, Z. officinale,* and *Z. officinale* Var Rubra, significantly attenuated the expression of MMP-2 activity in LPS-induced BPAE cells, suggesting their potential MMP-2 inhibitory activity may be applied for beneficial diet in terms of cardiovascular protection. However, searching for the potent bioactive compounds derived from Zingiberaceae rhizomes which responsible for inhibition of MMP-2 expression and its signaling mechanism in LPS-induced BPAE cells is still needed.

ACKNOWLEDGEMENT

This work was funded by Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia through Competency Grant (2012). We also thank Dr. Diah Iskandriati and Silmi Mariyah, MSc for sharing bovine artery endothelial cell collection at Laboratory of Microbiology and Immunology, Research Center for Primate, Bogor Agriculture University, Bogor, Indonesia.

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